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Trichoderma harzianum produces nonanoic acid, an inhibitor of spore germination and mycelial growth of two cacao pathogens

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Abstract

An isolate of *Trichoderma harzianum* Rifai from an infected cacao pod produces and secretes nonanoic (pelargonic) acid into a liquid culture medium. Nonanoic acid (NA) was very inhibitory to spore germination and mycelial growth of two cacao pathogens, *Crinipellis perniciosa* Stahel and *Moniliophthora roreri* Cif. H.C. Evans. It was highly active causing 75% inhibition of spore germination in an in vitro assay at a rate as low as 0.09 μM for *M. roreri* and 0.92 μM for *C. perniciosa*. Mycelial growth was comparatively less sensitive to inhibition, but still there was a 75% reduction in growth with 0.62 μM in *M. roreri* and 151 μM NA in *C. perniciosa*. In contrast, NA did not affect *Trichoderma* mycelial growth or spore germination at concentrations that were inhibitory to the pathogens. 6-pentyl-α-pyrone was also produced and secreted into the medium by *T. harzianum*, however; it was not antagonistic to the cacao pathogens. Although a number of metabolites produced by *Trichoderma* spp. have been identified in the past, this is the first report of NA production and secretion by any *Trichoderma*. The results suggest that NA may play a role in the successful use of some *Trichoderma* spp. isolates in the biocontrol of fungal diseases of plants.

Keywords: Antifungal activity; Biocontrol; Witches' broom disease; Frosty pod disease; *Crinipellis perniciosa*; *Moniliophthora roreri*; *Theobroma cacao*; Pelargonic acid

1. Introduction

The major constraints on cacao production are fungal diseases, which are estimated to cause about a 30% yield loss of cocoa beans worldwide. Two of the most destructive cocoa pathogens are *Crinipellis perniciosa* Stahel (= *Moniliophthora* [1]) and *Moniliophthora roreri* (Cif.) HC Evans. *C. perniciosa* causes witches' broom disease, which in Brazil alone has reduced production by more than 50% [2]. This disease attacks young developing leaves and the meristematic tissue of the flush shoots. Flowering cushions and young pods are also susceptible to infection, directly reducing crop yield. *M. roreri*, the causal agent of frosty pod rot, invades the fruits and destroys the developing seeds. Conidia are produced on the pod surface that can go on to infect newly developing pods. Both of these diseases are spreading rapidly in South and Central America, moving beyond their original sites of origin across natural geographical and ecological barriers [3].

Trichoderma species are common soil inhabiting fungi that have been developed as effective biocontrol agents against various phytopathogenic microorganisms including pathogens of cacao (*Theobroma cacao* L.) [4]. There is good evidence to support several mechanisms of disease control by *Trichoderma* species including mycoparasitism, competition, induced resistance and antibiosis [5]. *Trichoderma* species are known to produce over 40 different metabolites that may contribute to their mycoparasitic and antibiotic action [6]. We obtained several isolates of *Trichoderma* from a diseased cocoa pod that had fallen from a tree in a plantation in Ecuador. In this report, we identified nonanoic acid (NA) from the liquid culture media of a *Trichoderma harzianum* isolate from this pod, and we tested this compound for in vitro inhibitory activity against mycelial growth and spore germination of *C. perniciosa*

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and *M. roreri* to determine the biocontrol potential and possible mechanism of action of this fungus.

2. Materials and methods

The *T. harzianum* isolate was obtained from a cacao pod infected with *M. roreri* by Dr. Carmen Suarez, INIAP, Ecuador. The fungus was identified by Dr. Gary Samuels, USDA-Beltsville and a sample of this isolate will be available from ATCC. *T. harzianum* mycelium was grown on potato dextrose broth (PDB) in Erlenmeyer flasks at 25 °C on a shaker at 100 rpm for 2 weeks. The mycelium was filtered off, the pH of the filtrate lowered to 3–4 with 0.1 N HCl, and the filtrate was partitioned three times with ethyl acetate (EA). The combined EA fractions were evaporated, derivatized with BF_3-methanol and injected into a GC (HP 6890) equipped with a DB1701 capillary column (60 m × 250 μm × 0.25 μm, J and W Scientific, Folsom, CA, USA) and coupled to a HP 5973 mass selective detector. The temperature program was 70–240 °C at a rate of 10 °C/min after the first 2 min at 70 °C. Mass fragmentation was obtained by electron impact at 70 eV with an inlet temperature of 250 °C. The range scanned was 50–550 amu with a scan speed of 2.94 scans/s. The NA peak was identified using a NIST mass spectral library and confirmed by comparison with the authentic compound (Sigma, St. Louis, MO, USA).

Mycelial cultures of *C. perniciosa* and *M. roreri* were obtained from the American Type Culture Collection (ATCC#64190 and 64239, respectively). Mycelial plugs (5 mm) taken from actively growing cultures on potato dextrose agar (PDA) were inoculated on PDA containing different rates of NA (sterile-filtered and added to hot autoclaved agar before solidification) obtained commercially. Radial growth of the mycelium was measured over time. As a positive control, two commercial fungicides, azoxystrobin (Heritage, Zeneca Professional Products Inc., Wilmington, DE, USA) and propiconazole (Banner, Syngenta Crop Protection Inc., Greensboro, NC, USA) were compared with NA. Basidiospores of *C. perniciosa* were obtained from cacao broom cultures. Conidia from *M. roreri* were obtained from agar cultures. Droplets (100 μL) of spore suspension were transferred onto a PDA medium surface and germinated spores were counted after 24 h incubation at 25 °C using a microscope.

3. Results and discussion

The most widely studied antifungal compound produced by *Trichoderma* is 6-pentyl-α-pyrone (6-PAP) [7]. We also found 6-PAP in the liquid cultures of *T. harzianum*; however, it did not have an inhibitory effect on either of the two tested pathogens of cacao when they were grown in the presence of the compound (data not shown). The characteristic coconut aroma attributed to 6-PAP in the literature [8] was also not present in our *T. harzianum* cultures.

T. harzianum mycelium does produce 166 ± 26 ng/gdwt of NA in liquid cultures of PDB. NA was identified as the methyl ester by GC/MS with a retention time and mass spectra identical to the authentic compound: *m*/*z* (rel. int.), 172(1.4), 141(9.8), 129(12.6), 115(14), 101(5.6), 87(49.2), 74(100), 59(9.8), 55(15.4), 43(2.8).

NA strongly affected both mycelial growth and spore germination of the cacao pathogens. At an extremely low rate of 0.09 and 0.92 μM NA, 75% of spores were inhibited from germination in *M. roreri* and *C. perniciosa*, respectively (Fig. 1). Mycelial growth was comparatively less sensitive to inhibition, but still there was a 75% reduction in growth with 0.62 μM in *M. roreri* and 151 μM NA in *C. perniciosa* (Fig. 2). In comparative experiments using commercial fungicides, 1 μM azoxystrobin and 1 μM propiconazole inhibited mycelial growth of *M. roreri* by 47% and 75%, and for *C. perniciosa*, there was a 60% inhibition of mycelial growth with 1 μM azoxystrobin and 72% inhibition with 1 μM propiconazole (data not shown). On a molar basis, NA compared favorably with the commercial fungicides in the control of mycelial growth of *M. roreri*, but was not as effective against *C. perniciosa* as azoxystrobin or propiconazole. *T. harzianum* itself was quite insensitive to NA and very high concentrations, e.g. > 1 mM were needed to have an effect on either mycelial growth or spore germination (Figs. 1 and 2). Other fatty acids are known to be inhibitory to fungal growth. Walters et al. [9] found that the unsaturated fatty acids with 18–22 carbons inhibited mycelial growth in *C. perniciosa*, but a rate of 1 mM was required to reduce growth by 50%.

NA has been found in other fungi including *Aspergillus niger*, *Rhizopus stolonifer*, *R. oligoporus* and *Penicillium expansum* [10–12]. It has been suggested that NA may function as a natural self-inhibitor of spore germination. Uptake of NA by sporangiospores of *R. oligosporus* prevented the internal rise in pH that accompanies spore germination [10]. In these studies NA inhibited both

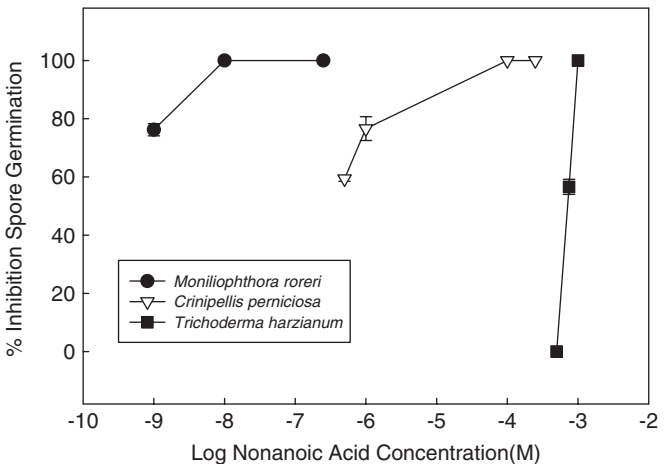

Fig. 1. Effect of nonanoic acid concentration on spore germination of *Moniliophthora roreri*, *Crinipellis perniciosa*, and *Trichoderma harzianum*. Control spore germination was 70% for *M. roreri*, 75% for *C. perniciosa* and 84% for *T. harzianum*.

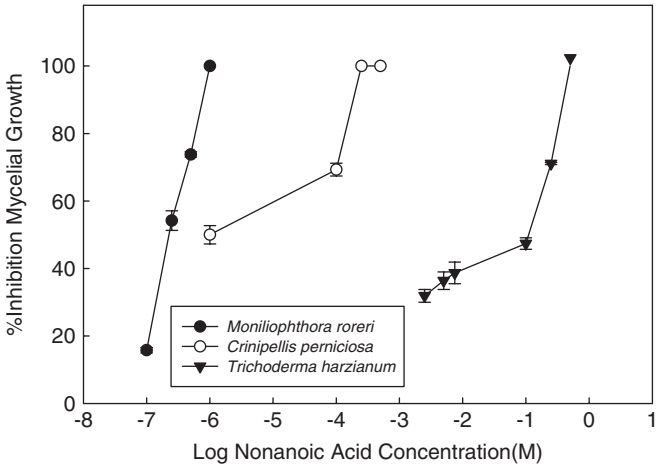

Fig. 2. Effect of nonanoic acid concentration on radial mycelial growth of *Monliophthora roreri*, *Crinipellis perniciosa*, and *Trichoderma harzianum*.

sporangiospore and conidia germination at concentrations of 1 mM. Our results support these findings and demonstrate that spores of *C. perniciosa* and *M. roreri* were inhibited at even lower concentrations (<1 μM). The effectiveness of NA at low rates in the present work is comparable to that of the fungicides trifloxystrobin and pyraclostrobin against *Cercospora beticola* [13] using a similar in vitro assay. In contrast, *Trichoderma* spore germination was not inhibited at a lower rate of NA. The insensitivity of *T. harzianum* to NA at rates below 1 mM might be due to its bioconversion to volatile metabolites via β-oxidation, esterification or decarboxylation. Kinderlerer [14] proposed this mechanism as a fungal strategy to remove lethal metabolites. Isolates from species from five genera (*Aspergillus*, *Penicillium*, *Trichoderma*, *Cladosporium* and *Fusarium*) are known to carry out these conversions [14].

The biosynthetic pathway leading to NA is not known. A possible pathway to the 9-carbon fatty acid may start with the amino acid threonine. Threonine is converted to 2-oxo-butyric acid by threonine dehydratase, which is oxidatively decarboxylated to propionyl-CoA. Propionyl-CoA serves as a three-carbon primer for fatty acid synthase to produce odd chain numbered fatty acids [15]. In fact, when the enzyme acetohydroxyacid synthase was inhibited in plants, raising the level of threonine and reducing the levels of branched chain amino acids, the predominant fatty acids in the sucrose polyesters of tomato trichomes had three, nine and 11 carbon chain lengths [16]. Identifying the biosynthetic routes to NA in *Trichoderma* would be useful both for understanding the regulation of NA production and for developing strategies to increase its synthesis and/or secretion. Given the significant inhibition of spore germination and mycelial growth of the cacao pathogens by NA, it would be useful to determine if the most effective *Trichoderma* isolates used as biocontrol agents against the cacao pathogens produce NA, and if NA, or a precursor, would be an effective addition to formulations of the biocontrol agents. Further research in these directions is in progress in our Laboratory. For example, we have found other strains of *T. harzianum* that produce NA as well as isolates of *T. paucisporum*, *T. theobromicola* and some of the *T. stromaticum* strains that are used for the biocontrol of witches' broom disease. The most commonly used products to control cacao diseases are the copper-based fungicides. These compounds are toxic to *Trichoderma* and it would be useful to determine if they could be replaced with NA, a antifungal compound more compatible with *Trichoderma*.

Although *Trichoderma* is known to be a common inhabitant of all soil types worldwide [17], these fungi may also be found growing endophytically in the bark of tropical trees [18], and the most commonly found species is *T. harzianum* [19]. Given the ability of *T. harzianum* to produce an effective antifungal compound against the cacao pathogens, and the discovery of this fungus growing avirulently within the cacao tree, if *T. harzianum* produces NA *in planta*, it may function as a natural symbiont of cacao protecting the plant from disease.

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